Biological insights from the direct measurement of purine release

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Abstract

Although purinergic signalling has been a well-established and accepted mechanism of chemical communication for many years, it remains important to measure the extracellular concentration of ATP and adenosine in real time. In this review I summarize the reasons why such measurements are still needed, how they provide additional mechanistic insight and give an overview of the techniques currently available to make spatially localised measurements of ATP and adenosine in real time. To illustrate the impact of direct real-time measurements, I explore CO_2 and nutrient sensing in the medulla oblongata and hypothalamus. In both of these examples, the sensing involves hemichannel mediated ATP release from glial cells. For CO_2 the hemichannels involved, connexin26, are directly CO_2-sensitive. This mechanism contributes to the chemosensory control of breathing. In the hypothamalus, specialised glial cells, tanycytes, directly contact the cerebrospinal fluid in the 3rd ventricle and sense nutrients via sweet and umami taste receptors. Nutrient sensing by tanycytes is likely to contribute to the control of body weight as their selective stimulation alters food intake. To illustrate the importance of direct adenosine measurements I consider the complex and multiple mechanisms of activity-dependent adenosine release in different brain regions. This activity dependent release of adenosine is likely to mediate important feedback regulation and may also be involved in controlling the sleep-wake state. I finish by briefly considering the potential of whole blood purine measurements in clinical practice.

Key Words:

ATP; Adenosine; Biosensor; Connexin; Purinergic

1.0 The biological imperatives for direct measurement of purine release

This article contributes to a special issue that celebrates the central role of Geoff Burnstock in originating and developing, with unparalleled energy over many decades, the field of purinergic signalling. Geoff was a highly inspiration speaker, and a compelling lecture from him, in which he surveyed the concepts that underpin purinergic signalling, inspired me to enter the field [1] and develop some of the methods for real-time measurement of ATP and adenosine that I outline in this article.

Our understanding of chemical neurotransmission originated with the identification of acetylcholine as a neurotransmitter. As part of the generalisation of the hypothesis of chemical neurotransmission to include additional potential neurotransmitters, several criteria necessary to establish rigorously whether a substance can act as neurotransmitter were articulated [2]: the release of the neurotransmitter by physiological stimuli (referred to as “collectability of the transmitter”); the synthesis and/or presence of the neurotransmitter in the presynaptic terminal; the ability of exogenous application of the proposed neurotransmitter to mimic the biological actions of the endogenous compound; and a mechanism to terminate the actions of the neurotransmitter.

At the time that Werman wrote his 1966 review [2], there was emerging evidence that favoured the existence of additional neurotransmitters such as dopamine, histamine, noradrenaline and GABA [3]. Although acetylcholine release had been demonstrated, satisfying the “collectability criterion” for the other emerging transmitters was regarded by Werman as “quite unlikely”. This led to experimental studies that concentrated on demonstrating the presence of the transmitter, and the identity of action of the proposed candidate with the endogenous transmitter. However, the stimulated release of ATP from nerve terminals had already been demonstrated by this time [4], as well as its identity of action with the endogenous transmitter [5]. Indeed, on the basis of careful pharmacology by Geoff Burnstock and colleagues, there

would soon be an appreciation that ATP could act via two different classes of receptor [6]. In retrospect it is therefore rather inexplicable that the evidence for ATP as a neurotransmitter was largely overlooked until the cloning of selective ionotropic and metabotropic receptors for ATP [7, 8]. Arguably it was the identification of these receptors that was the key development that moved the concept of purinergic signalling into the mainstream of physiology and neuroscience.

Given that purinergic signalling is now widely accepted, why has direct measurement of the release of purinergic signalling agents remained important in this field? The reasons for this stem from the complexity of purinergic signalling itself, both in the release of the key molecules, but also in their interconversion in the extracellular space [9-12] and their ability to act at multiple receptors [13].

ATP is very rarely released as a principal neurotransmitter in the nervous system [14]. This hints at one of the big unknowns for understanding ATP signalling: what are the cellular sources and mechanisms for its release? ATP receptors, both P2X and P2Y, are widely distributed in the central and peripheral nervous systems, begging the question “where does the ATP come from?”. As every cell contains millimolar concentrations of ATP, all cells are potential sources of ATP [15].

In fact, there is a multiplicity of ATP release mechanisms that include vesicular exocytosis [16], but also channel mediated mechanisms such as via connexin [17-20] or pannexin [21-23] hemichannels and other large conductance channels such as CalHM1 [24, 25]. Direct measurements of ATP release in real time can be extremely helpful in differentiating between possible mechanisms of release. Furthermore, ATP release can occur outside of conventional synaptic structures and can involve non-neural cells [26]. Direct measurements of ATP in the extracellular space can also assist in understanding the dynamics of its diffusion in tissue and,

the concentrations it reaches within the tissue. This latter point is important as different receptors are sensitive across different concentration ranges [27].

Werman identified an inactivating mechanism as an essential aspect of neurotransmission. In this regard purinergic signalling is highly complex: there are at least 4 classes of extracellular enzyme involved in the breakdown of ATP (the ecto-nucleoside triphosphate diphosphohydrolases, E-NTPDases; the ecto-nucleotide pyrophosphatases phosphodiesterases, E-NPPs; ecto-5'-nucleotidase; and the ecto-alkaline phosphatases) [10]. The E-NTPDases can generate extracellular ADP from ATP [9]. ADP referentially activates certain P2Y receptors. The inactivation process for ATP is thus an *activating* process for signalling via downstream purines, which not only include ADP but also adenosine. Prior release of ATP and its subsequent metabolism in the extracellular space is an important source of adenosine and the distribution of the enzymes that catabolize ATP are critical factors in its production [28]. To complicate matters still further, adenosine can be released directly from cells via equilibrative transporters [29, 30] and it may be released under some circumstances by exocytosis [31]. Adenosine acts at metabotropic receptors of its own, some of which are inhibitory [13, 32, 33]. There is thus the possibility that the largely excitatory actions of ATP/ADP can be limited or opposed by the inhibitory actions of adenosine that originates from this prior release.

Purinergic signalling thus has the capacity to be highly complex, with some surprising and perhaps counterintuitive aspects. To illustrate this, I have used the known properties of ectonucleotidases to calculate the diffusion of ATP and its breakdown products [34, 35] in a 2D-plane (Figure 1). This is an elaboration, in two dimensions, of a previous one-dimensional model presented in the context of purines potentially acting as morphogens ([36]). In this mathematical model, there is continual release of ATP from a point source with its simultaneous diffusion and conversion to ADP, AMP and adenosine. Several interesting predictions emerge from this model. Firstly, the diffusion of ATP is likely to be quite restricted

by its breakdown into ADP and downstream metabolites. Secondly, ADP will diffuse over a wider area and hence have a greater volume than ATP in which it could activate its cognate receptors. Even more dramatically, adenosine will diffuse much further away. While the actions of ATP, ADP and adenosine can overlap within this 2D plane, adenosine could in principle have actions remote from the location of the ATP release events from which it originated. Parenthetically, this would argue that detection of adenosine in the absence of ATP cannot be used as a sole criterion to support direct release of adenosine. Interestingly, this analysis suggests that adenosine can form a ring around the point source of the ATP release (Figure 1). This has the potential to provide spatial separation between the potentially excitatory actions of ATP/ADP and the potentially inhibitory actions of adenosine. A key factor that enhances the temporal and spatial complexity of purine interconversion is the feed-forward inhibition by ATP and ADP of the ecto-5'-nucleotidase that converts AMP to adenosine [35]. Previous analysis has suggested that this feed-forward inhibition gives temporal separation of the actions of ATP and adenosine [37, 38]. The new analysis presented here predicts that the feed-forward inhibition could substantially enhance the spatial separation between the actions of ATP and adenosine even although they originate from the same source. There is some experimental evidence that supports the prediction that adenosine can diffuse and have relatively distant actions from its point of origin. Tetanic stimulation of excitatory pathways in the hippocampus can cause adenosine receptor-mediated inhibition of a separate but non-stimulated pathway [39]. About half of this activity-dependent adenosine arises from prior release of ATP [30] (see also section 3.4.1).

The complexity and interdependence of signalling by ATP, ADP and adenosine, and the theoretical analysis that I have presented, indicate the value of direct, real-time measurement of the purines in the extracellular space, in helping to understand their temporal and spatial dynamics. Methods to directly measure purine release are likely to remain an important part of the toolkit required to unravel the physiological actions of purinergic signalling. In this review

I shall therefore consider the various methods for measuring ATP and adenosine release and the insights that have emerged from them.

2.0 ATP release

2.1 Luciferase-based

In remarkable work, Holton [4] was the first to demonstrate activity dependent release of ATP from sensory nerves. She achieved this by exploiting the firefly enzyme luciferase to document the presence of ATP in perfusate. Luciferase is a bioluminescent enzyme that metabolizes ATP in the presence of luciferin and oxygen to emit light. It has the advantage of being highly sensitive and selective for ATP. However, as an enzyme, luciferase has a very low turnover number. This means that it is very hard to obtain rapid dynamic information about ATP release and diffusion with this method. The amount of light emitted is very low, hence very sensitive cameras need to be employed and significant effort has to be expended to eliminate sources of stray light.

Nevertheless, luciferase has been used successfully *in vitro* to produce an iconic image of a spreading wave of ATP release from a lawn of cultured glial cells [40, 41]. In a technical tour-de-force luciferase has also aided the demonstration of permeation of ATP through connexin43 hemichannels [18]. More recently di Virgilio has produced a plasma membrane tethered extracellular luciferase (pmeLuc) [42-45]. This has enabled the distribution of ATP in whole animals to be examined. While not providing a dynamic picture, this methodology has given real advances in for example demonstrating the accumulation of ATP in tumours [45].

Development of luciferase-based ATP sensors remains the subject of much research. For example, there has been a recent report of bioluminescent resonance energy transfer (BRET) -based sensor (from a modified luciferase to a red fluorescent protein) that has the advantage of permitting ratiometric measurement of ATP [46]. These ratiometric measurements are

advantageous as they give a signal that is independent of the amount of the sensor expressed in tissue.

2.2 Other Imaging-methods

To develop alternative imaging methods for ATP that are technically easier to implement than luciferase and to obtain higher temporal resolution, several groups have developed fluorescent ATP sensors (Figure 2a). These utilise the epsilon subunit of the bacterial F_0F_1 ATPase as the ATP-sensing moiety. This is reasonably selective for ATP but will also bind ADP. In one version (iATPsnfr), the signal readout is via a circularly permuted GFP, however the sensitivity of this sensor is relatively low [47]. An alternative method has been reported (ATPOS), which employs organic fluorescent molecules bound to cysteine residues engineered into the ATP sensor (Figure 2a). This has better sensitivity than iATPSnfr but has significant cross reactivity with ADP and adenosine [48].

2.3 Sniffer based

Measurement of neurotransmitter release via its native receptors was first used to detect acetylcholine release from developing neurons [49, 50]. The same idea has been applied to measurement of ATP release either with isolated membrane patches [51] or using whole cells as the detecting element [22, 52, 53]. This method is best suited to *in vitro* measurements. For example, Roper and colleagues demonstrated the tastant evoked release of ATP from taste cells using this method [22, 53], and it has also been used to document spreading waves of ATP release in cortical slices [52]. We used this method to demonstrate ATP release, both spike-mediated and glutamate-evoked spike-independent, from spinal neurons and spinal cord tissue of the frog embryo [51]. Although we did not understand it at the time, the glutamate-evoked spike-independent ATP release may have originated from astrocytes, as this is now well known to occur [30].

2.4 Microelectrode biosensors

Electrochemical enzymatic biosensors based on oxidases offer a convenient fast responding and reasonably selective method for measuring a variety of small molecules in tissue. The basic design involves an oxidase enzyme, or a small cascade of enzymes, that can metabolize the analyte of interest and, in so doing, generate H_2O_2 in proportion to the analyte of interest.

Two types of enzymatic biosensor have been developed for ATP. In one design two enzymes, glycerol kinase and glycerol-3-phosphate oxidase (Figure 2b) are trapped on the biosensor surface [54]. If glycerol is provided at a level that saturates glycerol kinase (>0.5 mM), the reaction becomes solely dependent on the concentration of ATP. The product of the first step, glycerol-3-phosphate, is the substrate for the second, peroxide-producing, enzyme that gives an electrochemical signal proportional to ATP concentration. This sensor is insensitive to ADP and adenosine [54].

An alternative ATP detection cascade has been reported: hexokinase and glucose oxidase [55-57]. Here the hexokinase converts glucose to glucose-6-phosphate, which is not a substrate for glucose oxidase (Figure 2c). The sensing principle is thus a glucose-dependent competition between glucokinase and hexokinase, and the signal is manifested as a loss of the glucose oxidation current. While a large signal can be obtained, this biosensor necessarily has to be sensitive to variations in glucose concentrations around the K_m for glucose oxidase. This type of biosensor cannot provide measurement of ATP independent of glucose, which is a serious disadvantage *in vivo*, where local glucose concentrations may well change with time.

These enzyme cascades can be entrapped on the surface of a microelectrode that can be made in various dimensions, typically a few hundred μm long and 7-50 μm in diameter e.g. [25, 54, 58]. The enzymatic reaction at the surface of the electrode generates H_2O_2 and this can be detected electrochemically to give a current proportional to ATP concentration (Figure 2d).

2.5 New insights

The real value of direct ATP measurements lies in how they lead to new avenues of research and mechanistic understanding that goes beyond the simple observation of ATP in the extracellular space. To illustrate this, I give two related examples from my own research: ATP measurements in the context of CO_2 and nutrient sensing.

2.5.1 Control of breathing and identification of a new biological sensing molecule for CO_2

Breathing fulfils two essential purposes: the first is to provide oxygen required for the metabolic pathways that generate ATP. The complete metabolism of one molecule of glucose to CO_2 and H_2O during oxidative phosphorylation requires 6 molecules of O_2. Without this steady supply of O_2, ATP production would essentially cease with ultimately fatal consequences. But the complete metabolism of one molecule of glucose also produces 6 molecules of CO_2. This highlights the second essential purpose of breathing: removal of CO_2. Adult humans excrete about 20 moles (or ~880 g) of CO_2 per day. Were this to accumulate in the body, the pH of blood and other fluids would become highly acidic. Regulated excretion of CO_2 via breathing is thus an essential physiological function.

As might be expected, therefore, there are chemosensors that sense pH/PCO_2 and adjust the rate of ventilation maintain level of PCO_2 and pH in arterial blood within physiological limits. These sensors are found both peripherally in the carotid body, and centrally, within the brainstem. Peripheral chemosensors respond to changes in pH. The central chemosensors are located at the central surface of the medulla oblongata and were identified some 50 years ago. A consensus has emerged that changes in pH are a sufficient signal to regulate breathing i.e. the chemosensory cells detect changes in pH. While there are undoubtedly cells within the medulla that respond to pH particularly in the retrotrapezoid nucleus (RTN) [59] and medullary Raphe nuclei [60], there has also been long standing evidence for a direct effect of CO_2 on central chemosensors at the medullary surface [61, 62] that has been somewhat overlooked.

Our initial motivation to use ATP biosensors to study the central chemosensors came from the observation that the response of neurons to hypercapnia could be altered by ATP receptor antagonists [63, 64]. However, at which stage of the signalling process -from detection of pH/CO_2 to the adaptive change in breathing -ATP was involved, remained far from clear. We first placed ATP biosensors on the ventral surface chemosensory areas of the medulla in an artificially ventilated, anaesthetized rat, while monitoring breathing via the phrenic nerve. When the rat was made hypercapnic, we saw a rapid and localised increase in extracellular ATP that preceded any adaptive change in breathing [65]. This early release of ATP suggested that it could be released from the chemosensory cells themselves and play a causal role in the adaptive reflex. We gained initial evidence that this was the case through use of P2 receptor antagonists which greatly blunted the ventilatory response to hypercapnia [65].

We next demonstrated that CO_2-dependent ATP release could be recapitulated *in vitro* in an isolated slice of the ventral medullary surface [66]. Under these controlled conditions, ATP release was evoked by an increase in PCO_2 at constant extracellular pH (achieved by also increasing HCO_3^-) [66]. We were particularly interested in the possibility that the CO_2-dependent ATP release could be mediated via connexin hemichannels. An involvement of connexin26 (Cx26) was first suggested by its preferential localization in glial cells at the ventral medullary surface [66]. We gained further support from this hypothesis by documenting that a range of pharmacological agents that can act on connexins with some selectivity to Cx26 blocked the CO_2 dependent ATP release observed *in vitro* [66]. These same agents, when used *in vivo*, reduced the adaptive ventilatory response to CO_2 by a similar amount to the previously documented blockade of ATP receptors (~20%) and reduced the observed CO_2-evoked ATP release, supporting the involvement of Cx26 in the chemosensory control of breathing [66].

A fascinating possibility was that Cx26 might itself be CO_2 sensitive. We explored this hypothesis through expression of Cx26 in HeLa cells. Cx26 was sufficient to endow these cells with a CO_2-dependent conductance increase that was absent from parental HeLa cells [19]. The presence of Cx26 also permitted CO_2-dependent dye loading into, and CO_2-dependent ATP release from, HeLa cells [19]. We found that CO_2 could gate Cx26 in isolated membrane patches where pH could be controlled on both sides of the membrane suggesting that this was not an effect of pH [19]. The simplest interpretation of these data was that CO_2 has a direct effect on the gating of Cx26 and causes its hemichannels to open, thus permitting the ATP release.

To investigate possible mechanisms of CO_2 sensitivity, we exploited the fact that three closely related connexins (Cx26, Cx30 and Cx32) are sensitive to CO_2 [19] but that Cx31, with significant homology to Cx26, lacked sensitivity to CO_2. We hypothesized that CO_2 could carbamylate a lysine residue in the CO_2 sensitive connexins [19]. While there are several known examples of CO_2-carbamylation of proteins, the idea that this might be a general and important post-translational protein modification involved in physiological regulation was first proposed by George Lorimer [67].

Comparison of the sequences of Cx26, Cx30, Cx32 and Cx31, revealed a lysine residue and a motif specifically present in the CO_2 sensitive connexins [68]. An X-ray structure for Cx26 [69], which showed the residues of this motif and specifically that the lysine within the motif, K125, is oriented towards R104 in the neighbouring subunit of the hexamer. We proposed that carbamylation of K125 might allow formation of a salt bridge between this residue and R104 to form a “carbamate bridge” between subunits and induce conformational change in the channel. To provide support for this hypothesis, we simply transplanted the carbamylation motif into Cx31 and demonstrated that it gave a gain of CO_2 sensitivity [68]. Mutations of the residues K125 and R104 were then made to further test our hypothesis: for example, K125R

(arginine not being carbamylatable) destroyed CO_2 sensitivity in Cx26, as did R104A (removing the ability to make a salt bridge) [68]. While the mutational analysis strongly suggests carbamylation as a mechanism, it is not definitive. Recently however we have produced mass spectrometry evidence for the carbamylation of K125, and the consequent changes in channel gating in very high resolution cryoEM structures of Cx26 at different levels of PCO_2 [70].

This structural knowledge of how CO_2 binds to Cx26 has allowed us to develop a dominant negative subunit, dnCx26, to probe the role of CO_2 sensing via Cx26 in breathing. This subunit carries two mutations: R104A and K125R, which respectively prevent formation of intersubunit carbamate bridges and binding of CO_2. Homomers of dnCx26 are insensitive to CO_2 and dnCx26 efficiently assembles into hexamers with wild type Cx26 [71]. Heteromeric dnCx26-wtCx26 hemichannels are not sensitive to CO_2 sensitivity showing that dnCx26 acts as a dominant negative subunit to remove CO_2 sensitivity from the endogenously expressed wild type Cx26 [71]. By means of lentiviral constructs, we have driven selective expression of dnCx26 in glial cells at the ventral medullary surface (Figure 4). When dnCx26 was expressed caudally in a small area called the caudal parapyramidal area (cPPy) it altered the CO_2 sensitivity of breathing [71]. Expression of dnCx26 reduced the change in tidal volume and minute ventilation by about 33% compared to expression of the Cx26 wild type subunit (as a control). There are highly pH-sensitive serotonergic cells in the cPPy and these project to other nuclei involved in the control of breathing [72, 73]. CO_2 detection mediated by superficial glial cells that express Cx26, could thus converge with pH detection mediated by colocated serotonergic neurons in the cPPy via CO_2-regulated ATP release [71]. As central chemoreceptors mediate about 70% of the adaptive response to hypercapnia, CO_2 sensing via Cx26 in the caudal parapyramidal area contributes nearly half of this response to modest levels of hypercapnia. Previous studies have suggested that pH- and direct CO_2-sensing each contribute about 50% to the chemosensory regulation of breathing via central chemosensors

[61]. This suggests that the small population of Cx26-expressing glial cells in the cPPy may therefore mediate the majority of direct CO_2-mediated control of breathing (Figure 4).

While the preceding account has concentrated on the role of Cx26 in glia, it is important to note that direct pH sensing by glia of the RTN may also be important. pH-stimulated ATP release has been described and activation of RTN glia will enhance breathing via an ATP receptor-dependent mechanism [74, 75].

2.5.2 Nutrient sensing in the hypothalamus

We have known since the demonstration that lesions in specific areas of the brain can lead to obesity or anorexia [76, 77], that food intake and energy expenditure are controlled by the brain. The arcuate nucleus of the hypothalamus contains opposing neural circuits that promote food intake and energy conservation (orexigenic) and diminish appetite and increase energy expenditure (anorexigenic). Arcuate neurons integrate both peripherally derived signals (such as the hormones leptin, ghrelin and circulating nutrients such as glucose and fatty acids) and centrally derived signals such as orexin. The arcuate nucleus is a medial and ventral structure that is adjacent to the 3rd ventricle and median eminence. Tanycytes are glial cells within the wall of the 3rd ventricle [78]. They may fulfil a variety of functions such as forming a barrier between the CSF of the 3rd ventricle and the parenchyma, and transport of leptin from the CSF to the parenchyma [79-82]. At least some tanycytes appear also to be neural stem cells [83-86]. There has been a longstanding hypothesis that tanycytes sense nutrients, such as glucose [87]. This was based on the observation that the components of glucosensing found in pancreatic beta cells are also present in tanycytes: glucose transporters, glucokinase, K-ATP channels. We have utilised a combination of Ca^{2+} imaging and ATP biosensing to directly measure the responses of tanycytes to glucose in *in vitro* brain slices [88].

We found that tanycytes were highly sensitive to glucose provided it was applied selectively to the cell body, and not uniformly over the whole slice [88]. Glucose evoked a Ca^{2+} wave that travelled across the layer of tanycyte cell bodies. The propagation of this Ca^{2+} wave depended on the release of ATP acting via P2Y1 receptors [88]. We demonstrated the glucose-dependent release of ATP from tanycytes with biosensor measurements [88]. The first hint that tanycyte glucosensing might differ from that of pancreatic beta cells came from our observation that non-metabolisable glucose analogues could also activate Ca^{2+} signals in tanycytes. This led us to test whether a G-protein coupled receptor could mediate at least some of the glucose responses. The most obvious receptor to test was the sweet taste receptor originally described in the taste buds of the tongue. We found that tanycytes could respond to sweet tastants [89] and that this was mediated via $P2Y_1$ receptors. Deletion of the sweet taste receptor (the tas1r2-tas1r3 heterodimer) abolished the glucose response in about 60% of tanycytes, but in the remainder glucose evoked responses were still recorded [89]. This suggested the presence of more than one glucosensing mechanism. This latter mechanism may indeed involve glucokinase and K-ATP channels, as this has been described as underlying glucose responses in cultured tanycytes [90]. These same authors have shown that the Ca^{2+} waves evoked by glucose in cultured tanycytes depend on Cx43 hemichannels [90].

The presence of the sweet taste receptor in tanycytes suggested that other taste receptors might be present too [91, 92]. We were especially interested in receptors for amino acids for two reasons. Firstly, although there are multiple types of umami receptor (taste of amino acids), one type is closely related to the sweet taste receptor: it consists of a heterodimer of the tas1r1 and tas1r3 subunits (i.e. shares a common subunit with the sweet taste receptor). Secondly, amino acids are powerful determinants of satiety and injection of amino acids into the brain [93], particularly the mediobasal hypothalamus reduces food intake [94]. We therefore applied our methods to test whether tanycytes respond to amino acids and found that a range of amnio acids evoked Ca^{2+} waves in tanycytes. Once again, the amino acids

needed to be applied selectively to the cell body of the tanycytes to evoke responses [25]. The amino acid evoked responses were ATP receptor dependent, but unlike the glucose responses they depended on multiple P2 receptor subtypes. Although the $P2Y_1$ receptor was involved, use of $P2Y_1R$ antagonists by themselves were insufficient to block amino acid induced Ca^{2+} waves. Complete blockade was only achieved by a combination of MR2500 (selective $P2Y_1R$ antagonist) and non-selective P2 antagonists such as PPADS and suramin [25]. Using ATP biosensors, we recorded amino acid induced ATP release (Figure 4), and found that this induced a spreading wave of ATP from the tanycyte cell body layer into the parenchyma at a speed of about 3.6 μm/s [25]. At least two different receptors mediated these amino acid responses in tanycytes. Responses to arginine and lysine were mediated via the tas1r1/tas1r3 heterodimer and were abolished in female tas1r1 knockout mice. By contrast responses to alanine were unaffected by the deletion of the tas1r1 gene. Instead, alanine responses were mediated via mGluR4 receptors. ATP release was channel mediated, but different amino acids recruited different release pathways: arginine appeared to evoke ATP release via pannexin-1, whereas alanine evoked ATP release via CalHM1 [25] (Figure 5).

A key question is whether tanycytes can communicate with neurons to pass on information about nutrient levels in the CSF. We have used optogenetic methods to demonstrate that activation of tanycytes will excite neurons in both the orexigenic and anorexigenic pathways of the arcuate nucleus [95]. This excitation was at least partly mediated via ATP and activation of P2 receptors. Crucially optogenetic activation of tanycytes caused a short-lasting increase in food intake [95]. The challenge now is to understand the dynamics of how nutrients change in the CSF following food intake, and how natural stimuli (as opposed to optogenetic stimuli) alter the activation of arcuate neurons and the intake of food.

3.0 Adenosine release

Historically, extracellular adenosine has been measured via microdialysis combined with offline HPLC analysis. While this method works and for example showed the accumulation of

adenosine during wakefulness [96], it is highly laborious and lacks temporal resolution. Furthermore, the microdialysis probes are quite large and implanted, they become encapsulated by a reactive glial scar [97]. This means that microdialysis does not properly sample from the extracellular space [98] and cannot give an absolute concentration of adenosine but instead reports relative change in concentration.

3.1 Microelectrode biosensors

Biosensors for adenosine utilize the enzymes that are involved in processing purines: adenosine deaminase, purine nucleoside phosphorylase and xanthine oxidase [38]. This cascade successively converts adenosine via inosine, hypoxanthine and xanthine to uric acid, and in so doing results in the production of H_2O_2 in proportion to the adenosine concentration (Figure 6a). A biosensor based on 3 enzymes, will be sensitive to substrates of all of the enzymes. A specific adenosine signal can be abstracted by making differential measurements between a biosensor having all three enzymes, and one lacking adenosine deaminase (and thus insensitive to adenosine). An alternative strategy is to compare adenosine biosensor recordings made under control conditions to those made in the presence of an adenosine deaminase inhibitor such as coformycin or EHNA. Adenosine microelectrode biosensors have a rapid temporal resolution (seconds) successfully shown the activity dependent production of adenosine from spinal cord during motor pattern generation [38]; release of adenosine during metabolic stress such as hypoxia or glucose/oxygen deprivation [99-101]; and activity dependent release both directly, and as a result of prior release of ATP followed by conversion in the extracellular space [30, 31, 102-104].

3.2 Direct electrochemistry

A more recent alternative to biosensing has been provided by direct electrochemical detection of adenosine [105-109]. By using carbon fibre microelectrodes and fast scan cyclic voltammetry (FSCV), the specific redox peaks in the voltammogram linked to the oxidation and reduction of adenosine on the microelectrode surface can be detected. These peaks can

be differentiated from those of other purines such as inosine, hypoxanthine allowing selective detection of adenosine. The temporal resolution of this method is of the order of 100s of milliseconds and is limited by the rate at which the microelectrode can be scanned -typically on the order of 400 Hz. This method has been used to document activity dependent adenosine release and spontaneous adenosine transients [108-110]. It has helped to document a link between adenosine release and dilation of striatal blood vessels [111]. Reassuringly, the conclusions derived from FSCV and biosensor measurements are highly consistent.

3.3 Imaging methods

Very recently imaging methods have been developed for adenosine and other modulatory transmitters. These are based on engineering a GPCR for the modulator in question to have a circularly permuted GFP in place of one of the intracellular loops -a so-called GRAB (GPCR activation-based) sensor. Binding of the ligand changes the fluorescence intensity of the GRAB sensor. GRAB sensors have been devised for dopamine and norepinephrine [112, 113], and most recently adenosine [114]. $GRAB_{Ado}$ is highly sensitive to adenosine (in the nM range) but loses linearity above 1 μM. Furthermore, although it is most sensitive to adenosine, it has significant cross-reactivity with ADP and ATP (in the 100s of nM to low μM range) at concentrations which are likely to be present during biological signalling. Indeed, if the predictions of Figure 1 are correct, ATP and ADP may be present at considerably higher concentrations that adenosine itself. Thus, although $GRAB_{Ado}$ is an exciting development its characteristics are not yet ideal to detect adenosine selectively over the concentration range that it is likely to operate in CNS (low μM).

3.4 New Insights

3.4.1 Activity dependent adenosine release in cerebellum and hippocampus

Pioneering work from Dunwiddie's group provided the first evidence for activity-dependent adenosine release in the hippocampus [39]. In elegant work, they stimulated two independent excitatory synaptic pathways in area CA1, and demonstrated that prestimulation with a brief

tetanus of one pathway could inhibit synaptic transmission in the other by an adenosine receptor-dependent mechanism. This showed not only the activity dependent production of extracellular adenosine, but also that it was capable of diffusing to affect neighbouring synapses in a manner akin to the predictions of Figure 1.

Direct measurement of adenosine release via microelectrodes, as opposed to inferring its presence via pharmacological effect, has been advantageous in working out mechanisms of its production in the extracellular space. Our studies have found multiple mechanisms for activity dependent adenosine release and shown that it is Ca^{2+}- and TTX-dependent [30, 104] and can be modulated via metabotropic receptors [104, 115]. In hippocampus, a fast component of adenosine release (approximately 40%) occurs via equilibrative transporters from neurons [30]. This component of adenosine release is consistent with the observation that feedback inhibition of activity in the CA1 area of the hippocampus is independent of extracellular metabolism of previously released ATP [29] (Figure 6b). However, matters are more complex than might be imagined from this previous work as deletion of ecto-5'nucleotidase (CD73) shows that around half of extracellular adenosine also arises from prior release of ATP and conversion in the extracellular space [30] (Figure 6b). This indirect pathway for adenosine production depends upon release of ATP from astrocytes and can be blocked by genetic or pharmacological targeting of astrocytes [30]. In striatum, activity dependent adenosine release has also been reported [108] and at least some of the enzymes that mediate production of extracellular adenosine are located on microglia [116]. Activity dependent adenosine release, which is phenomenologically very similar to that in the hippocampus, also occurs in the cerebellum when the parallel fibres are stimulated [104]. In cerebellum there is also a similar division between the direct release of adenosine, and its production via the breakdown of ATP in the extracellular space [31]. However, in cerebellum we obtained evidence that adenosine might be release via exocytosis: an inhibitor of the vesicular proton pump, bafilomycin, greatly reduced the direct release of adenosine [31].

3.4.2 Glutamate receptor-evoked adenosine release and links to the control of sleep

In both cerebellum and hippocampus at least some of the activity-dependent adenosine release is glutamate receptor-dependent and can be reduced by antagonism of AMPA and NMDA receptors [30, 31]. In hippocampus a large proportion of the glutamate-evoked adenosine release is Ca^{2+} dependent [30]. We have studied glutamate-evoked release in the basal forebrain and found that it can be stimulated by both NMDA and AMP receptors (as well as other excitatory neurotransmitters such as orexin, histamine and acetylcholine) [117]. We found that here, unlike in the hippocampus, the adenosine release was unaffected by removal of Ca^{2+} and instead depended on extracellular Na^+ [118]. AMPA-evoked adenosine release in the basal forebrain demonstrated a dependence on time of day, could be enhanced by sleep deprivation and following sleep deprivation had a dependence on iNOS [117]. This parallels the demonstration *in vivo* that induction of iNOS during sleep deprivation contributes to the enhanced adenosine levels in the basal forebrain [119, 120]. Interestingly our investigations showed a link between AMPA receptor-evoked adenosine release and neural energy consumption. By using ouabain, an inhibitor of the Na^+-K^+ exchange pump, we showed that adenosine release depended on activation of this pump [118] (Figure 6c). The Na^+ influx from action potential firing and AMPA receptor stimulation activates the Na^+-K^+ exchange pump, which because it consumes ATP, creates intracellular adenosine which then crosses the membrane, presumably via equilibrative transporters [118]. Our demonstration that adenosine release depends on ATP utilisation by the Na^+-K^+ exchange pump, links to understanding of the energy budget in grey matter: here about 80% of ATP consumption is predicted to be linked to pumping Na^+, which has accumulated intracellularly as a result of electrical and chemical signalling, back out of the cell [121] (Figure 6d). This in turn links to the hypothesis that local energy depletion in the basal forebrain leads to accumulation of adenosine and the onset of sleep [122]. We would modify this hypothesis by suggesting that elevated rates of neuronal activity, and resulting Na^+ entry, lead to enhanced consumption of ATP during wakefulness and hence an accumulation of extracellular adenosine. It is interesting in this context that activity of glutamatergic neurons in the basal forebrain seems to contribute to the

accumulation of extracellular purines [114], as their local activity and release of glutamate within the basal forebrain could trigger the types of mechanism that we have described in our *in vitro* work.

4.0 Clinical applications

The observation that the purines, adenosine, inosine and hypoxanthine are released from ischaemic tissue means that measurement of these metabolites might have clinical utility in the diagnosis of ischaemic pathologies such as stroke [123-126], myocardial infarction [127] and neonatal hypoxia-ischaemia [128-130]. One barrier to the routine measurement and use of purines as a diagnostic aid in medicine, is the difficulty in measuring them. The turnover of adenosine in blood is of the order of seconds [131], and even the further downstream metabolites inosine and hypoxanthine have a turnover times of minutes. While this can be partially avoided by sample treatment (cooling, separating the plasma and adding a cocktail of blockers to stop uptake and degradation), the elaborate procedures required have prevented adoption of purine measurements in clinical practice. Our invention of purine biosensors and the development of a biosensor array that allows the rapid determination of purines in whole, unprocessed blood within minutes of sampling potentially removes this barrier [126, 132-134].

By exploiting carotid endarterectomy, a procedure used to lessen the risk of stroke, as a model in which a mild but timed ischaemic insult is delivered to the brain, we have shown that purines are a very sensitive indicator of brain ischaemia in human patients [123]. Further studies have shown that purine levels are elevated in the venous blood of stroke patients compared to healthy controls and patients with mimicking conditions [126]. A similar assay has been used to show the rapid elevation of purines in the blood of patients suffering from myocardial infarction [127]. As ischaemia is a condition that attends many pathologies, it is unlikely that purines by themselves will be a diagnostic. However, in conjunction with clinical examination and suspicion, whole blood purine measurements are likely to be an excellent screening tool

to rapidly rule in or out serious medical emergencies such as stroke or myocardial infarction, thus prioritising patients for highly resourced specialised treatments that can be transformative for long term outcomes.

Measurement of purines could also be important for recognition of non-ischaemic conditions. A promising avenue for application of purine measurements is in the field of epilepsy. Many studies have shown release of both ATP and adenosine during seizure activity both *in vitro* and *in vivo* [135-138]. In neurological conditions such as depression there is evidence that the concentrations in plasma/serum of ATP metabolites such as adenosine, inosine and hypoxanthine are altered [139]. There is also emerging evidence that disorders of purinergic signalling may be associated with several different neurological disorders [140-144]. Nevertheless, it remains to be established whether measurement of purines in these contexts would have clinical diagnostic value.

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Declarations of Interest

The author is the founder of, and holder of equity in, Sarissa Biomedical Ltd, a company that commercialises the biosensors that are mentioned in this article. He is also a named inventor on patents held by Sarissa Biomedical Ltd.

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Figure Legends

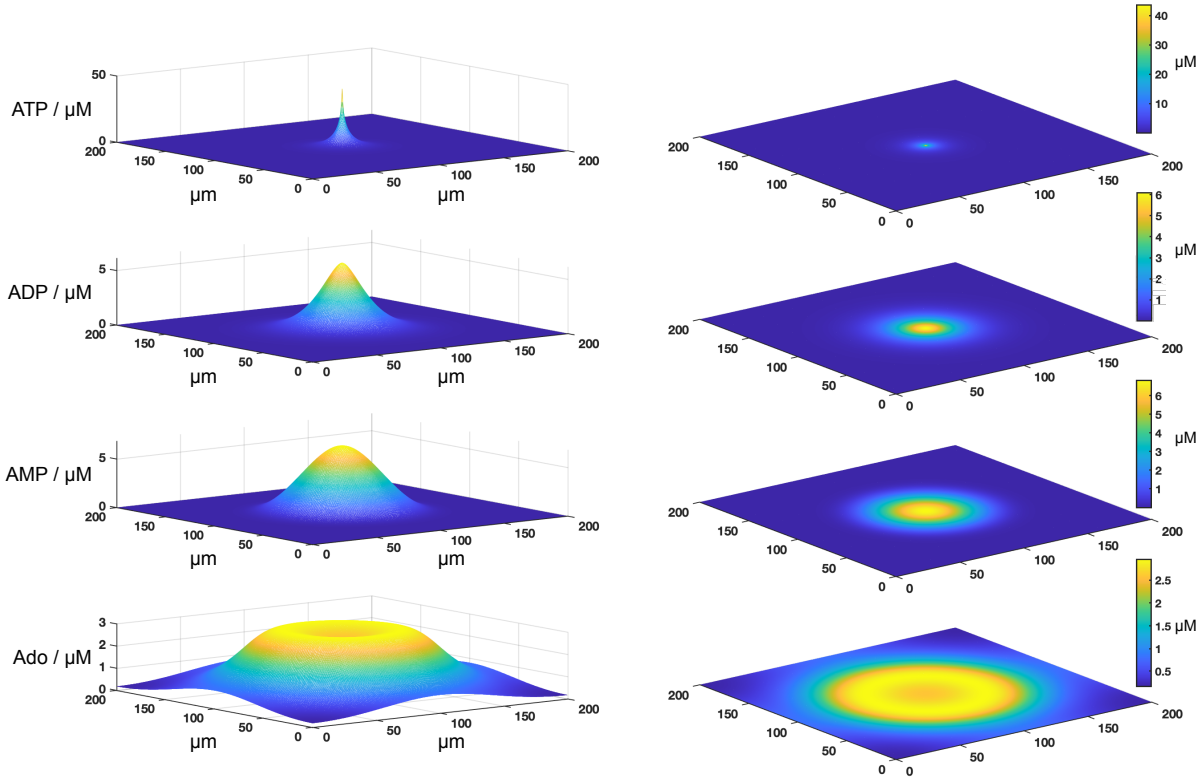

Figure 1. Simulation of diffusion and metabolism of ATP in 2 a dimensional plane originating from a central point source of ATP release after 5s. The left panel shows the profile of purine concentrations in 3 dimensions, while the right panel shows the same data but in two dimensions. Notice that elevated concentrations of adenosine are present across a large portion of the 2-D plane but that ATP is highly restricted. Owing to the feedforward inhibition by ATP and ADP of the conversion of AMP to adenosine, a ring of elevated adenosine levels forms round the point source of ATP but is physically separated from it. This analysis predicts that it is likely that adenosine arising from prior release of ATP in tissue will often be detected in the absence of any elevated ATP levels.

The conversion of ATP and metabolites are described by Michaelis-Menten kinetics (based on literature values [34, 37, 38]: ATP, K_m 33.3 μM, V_{max}, 100 μM/s; ADP, K_m 9.5 μM, V_{max} 20 μM/s; AMP, K_m 0.94 μM, V_{max}, 20 μM/s; both ATP and ADP inhibit the conversion of AMP to

adenosine (Ado) with a IC_{50} of 0.1 μM. The simulation is based on the following parallel linked partial differential equations: $\partial \mathbf{C_{ATP}} / \partial t = D \nabla^2 \mathbf{C_{ATP}} - k_1 \mathbf{C_{ATP}}$; $\partial \mathbf{C_{ADP}} / \partial t = D \nabla^2 \mathbf{C_{ADP}} + k_1 \mathbf{C_{ATP}} - k_2 \mathbf{C_{ADP}}$; $\partial \mathbf{C_{AMP}} / \partial t = D \nabla^2 \mathbf{C_{AMP}} + k_2 \mathbf{C_{ADP}} - k_3 . k_i \mathbf{C_{AMP}}$; and $\partial \mathbf{C_{ADO}} / \partial t = D \nabla^2 \mathbf{C_{ADO}} + k_3 . k_i \mathbf{C_{AMP}}$. Where D is the diffusion coefficient (300 $\mu m^2/s$), ∇^2 is the double differential with respect to distance in the x and y dimensions, **C** is a matrix for the concentration of the subscripted analyte (in x and y dimensions) and k_1, k_2 and k_3 are rates based on the Michaelis-Menten kinetics given above. $k_i = IC_{50} / \{IC_{50} + \mathbf{C_{ATP}} + \mathbf{C_{ADP}}\}$. These equations were solved numerically using code written for Matlab.

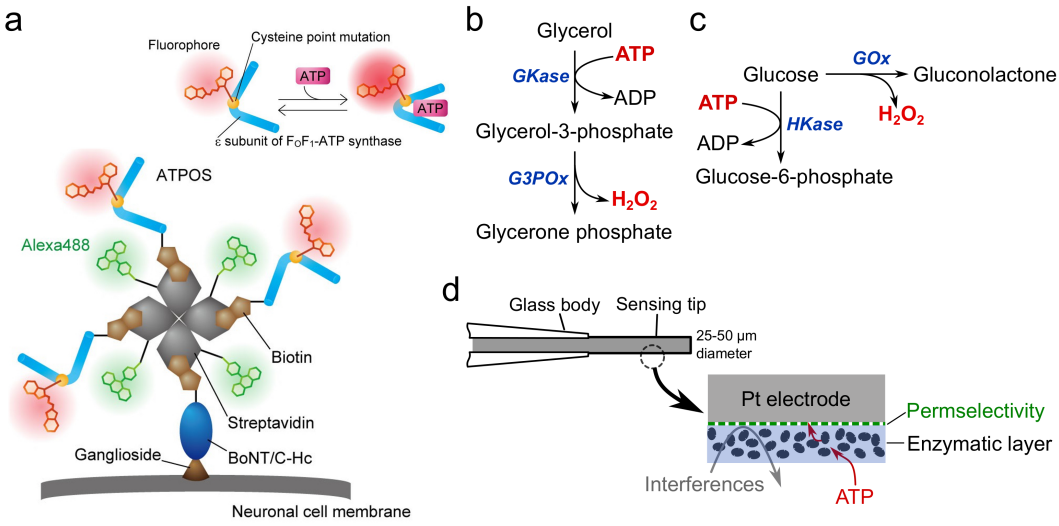

Figure 2. Methods for real time measurement of ATP. a) Fluorescent biosensor based upon the ε subunit of the F_0F_1 ATPase. Inset: Binding of ATP to this subunit causes conformational change and an increase in fluorescence of a fluorophore attached to the protein via a cysteine residue. Main diagram: assembly of the ATP sensor onto Alexa488 labelled streptavidin allows ratiometric measurement of ATP at two different wavelengths. The entire assembly can be anchored to the outer surface of neurons via the C-subunit of botulinum neurotoxin. (Diagram from [48]). b) Enzymatic cascade for the detection of ATP

via glycerol kinase (GKase) and the proportional production of H_2O_2 by glycerol-3-phosphate oxidase (G3POx). Note glycerol needs to be provided for this sensor to work. c) An alternative cascade which utilizes the competition for glucose between glucose oxidase (GOx) and hexokinase (HKase). Note that this biosensor is sensitive to glucose and ATP, and the signal results from a drop in the rate of production of H_2O_2. d) Schematic diagram of a microelectrode biosensor. The enzymatic cascade for ATP production is trapped in a thin layer on the surface of the sensing tip. The rate of production of H_2O_2 from the entrapped enzymes can be detected electrochemically by the Pt electrode, usually poised at a potential of +500 to 600 mV with respect to an Ag/AgCl reference. A permselective coating over the surface of the Pt electrode but beneath the enzymatic layer prevents interferences such as ascorbate or urate from reaching the microelectrode surface and thus giving a non-selective signal, while still permitting access of H_2O_2 and hence selective detection of ATP.

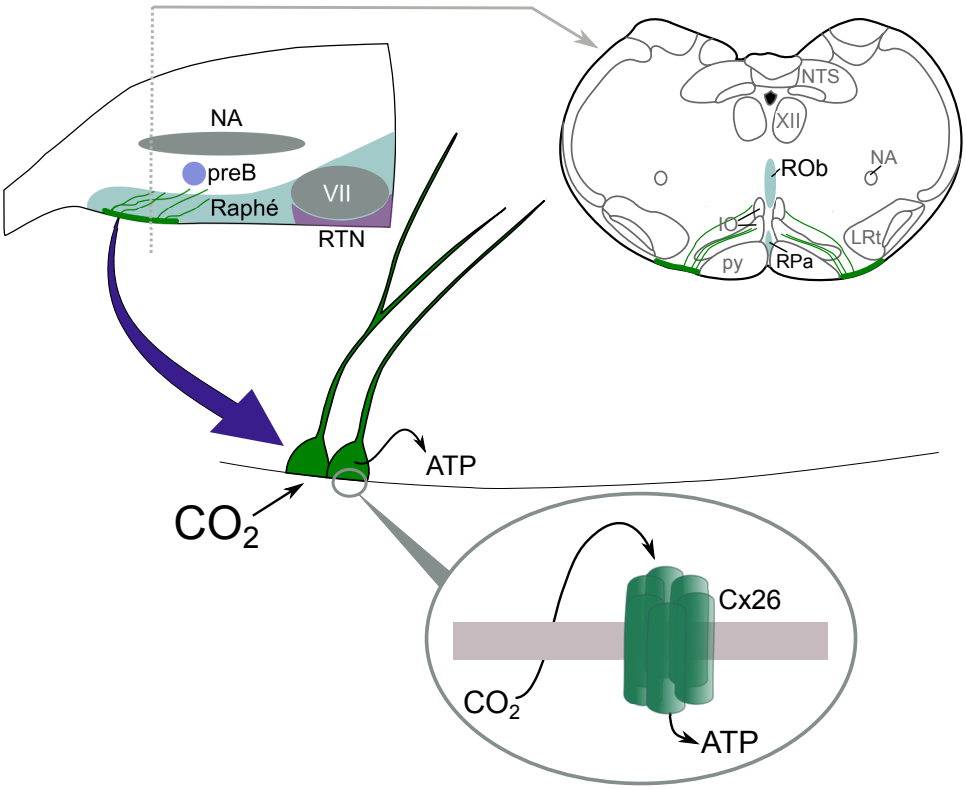

Figure 3. Direct sensing of CO_2 via Cx26 in the medulla oblongata. Conceptual summary of findings from [19, 65, 66, 68, 71]. Cx26 is expressed in glia at the very ventral surface of the medulla oblongata in the caudal parapyramidal area (shown in green in anatomical maps, parasagittal and coronal on the left and right respectively). These glial cells have dorsally directed processes. CO_2 crosses the plasma membrane to bind on an intracellular motif in Cx26 to open the hemichannel and allow the release of ATP. The ATP triggers an increase in breathing. NA, nucleus ambiguus; preB, preBötzinger complex; VII, facial (7^{th}) nucleus; RTN, retrotrapezoid nucleus; IO, inferior olive; ROb, Raphe obscurus; RPa, Raphe pallidus; LRt, lateral reticular nucleus; py, pyramids; NTS, nucleus tractus solitarius; XII, hypoglossal (12^{th}) nucleus.

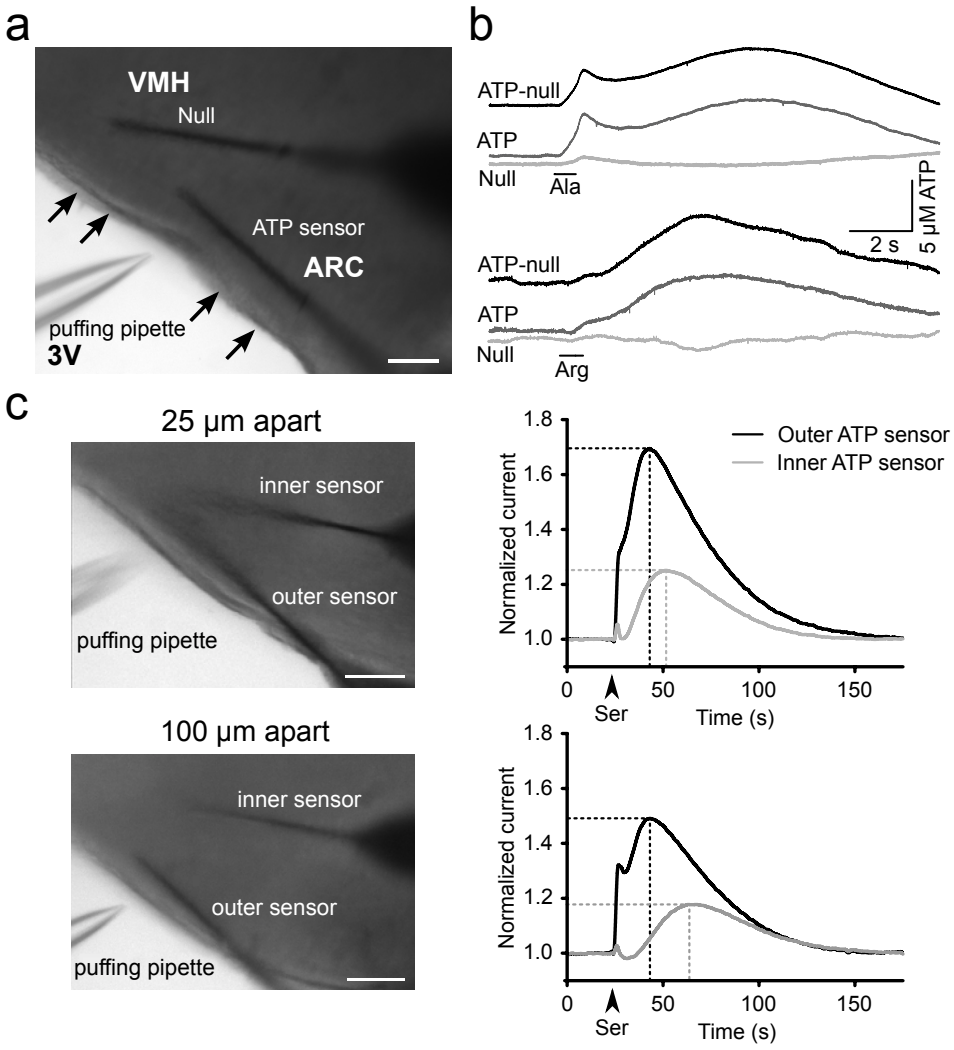

Figure 4. Amino acid evoked ATP release from hypothalamic tanycytes. a) Image of hypothalamic brain slice. The arrows point to the layer of tanycyte cell bodies at the interface between the 3rd ventricle (3V) and the arcuate nucleus (ARC) and ventromedial hypothalamic nucleus (VMH). An ATP biosensor and a null sensor (identical to ATP sensor but lacking the ATP-detecting enzymes) have been inserted into the slice close to the tanycyte cell bodies. These sensors have been fabricated on 7 µm carbon fibre microelectrodes. A patch pipette is close to the tanycyte cell bodies to allow puffs of amino acids selectively onto their cell bodies. b) Alanine and arginine puffs evoked a large signal on the ATP biosensor but not the null sensor, indicating the release of ATP. c) Insertion of two ATP biosensors at different distances from the tanycyte layer shows that a wave of ATP release that propagates into the parenchyma. Scale bars are 100 µm. Reproduced from [25].

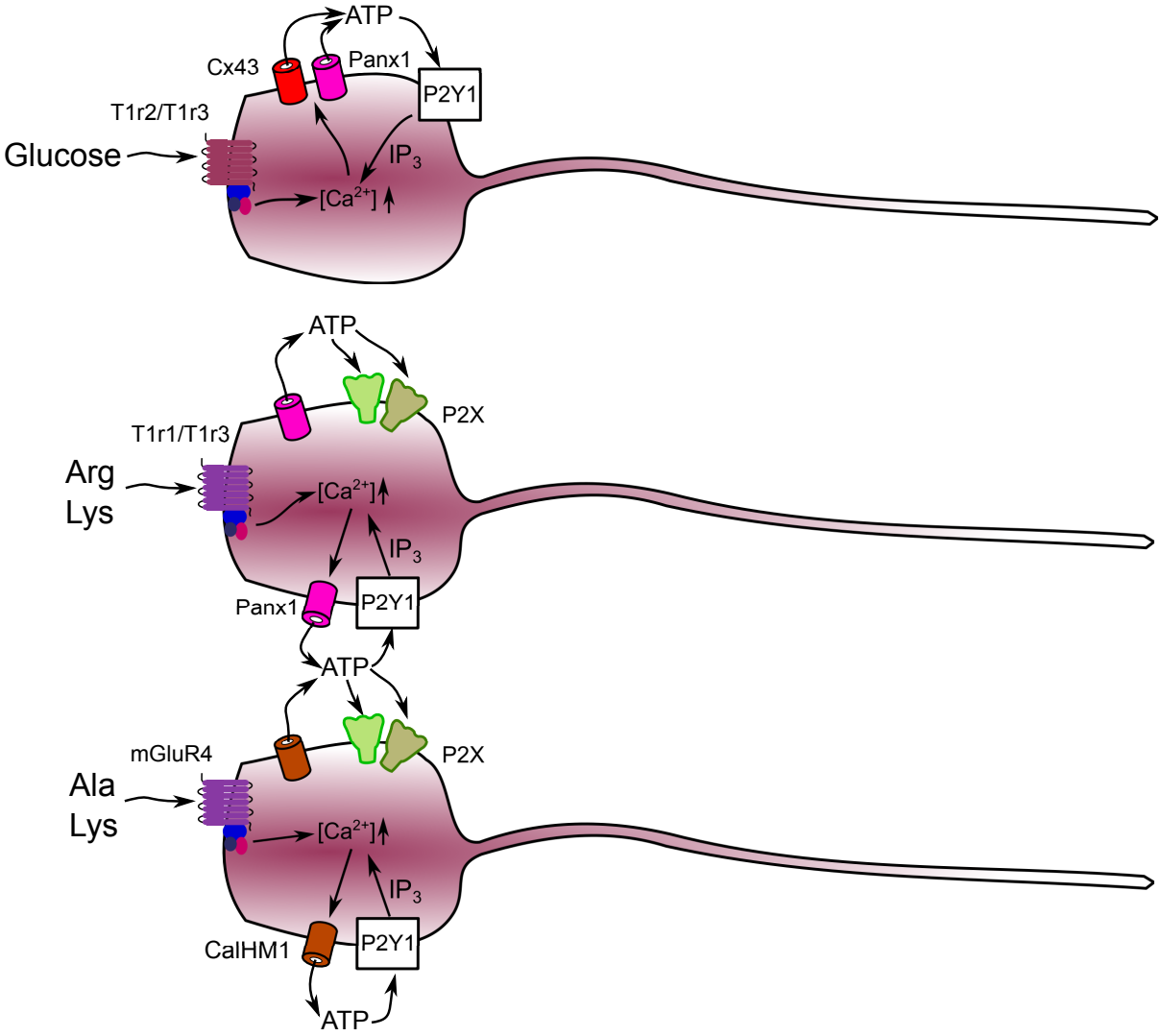

Figure 5. Nutrient sensing by hypothalamic tanycytes. Conceptual summary of findings from [25, 88-90].Tanycytes utilize a series of taste receptors to detect glucose, and amino acids such as alanine, arginine, and lysine. In all cases the detection involves hemichannel mediated ATP release and subsequent propagation of ATP receptor dependent Ca^{2+} waves. For each nutrient however the ATP release pathways and P2 receptors involved in signal propagation differ.

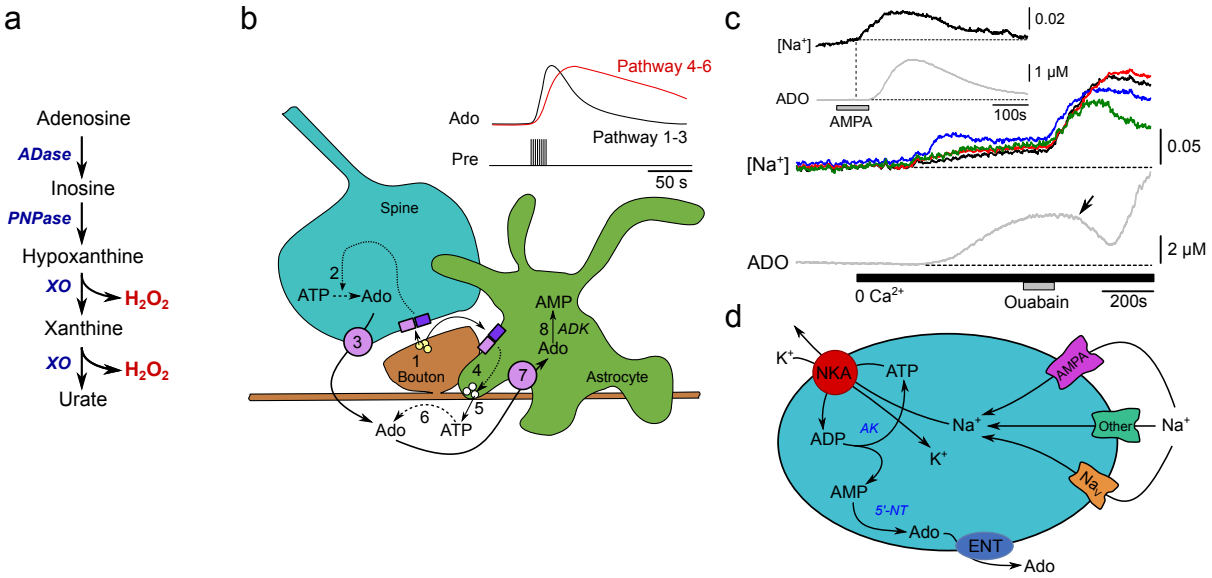

Figure 6. Mechanisms of activity dependent adenosine release revealed by adenosine biosensor measurements. a) Enzymatic detection cascade for adenosine and related purines. b) Scheme for two parallel pathways for adenosine release or production in the extracellular space following activation of the Schaffer collaterals in area CA1 of the hippocampus (shown brown). Pathway 1-3 is neuronal and involves synaptic release of glutamate (1) activation of postsynaptic NMDA and AMPA receptors, increased consumption of ATP and hence production of intracellular adenosine (2) which then diffuses out of the cell via equilibrative transporters (3). Pathway 4-6 represents is astrocytic and involves glutamate receptor stimulated exocytotic release of ATP (4,5), followed by conversion to adenosine in the extracellular space (6). Note that the kinetics of the two pathways are distinct (inset). Reproduced from [30]. c) In basal forebrain, AMPA-evoked adenosine release is Na^+ dependent. AMPA evokes a rapid increase in intracellular Na^+ followed by delayed adenosine release. An increase in intracellular Na^+ and subsequent adenosine release can also be triggered by removal of extracellular Ca^{2+}. Application of an inhibitor of the Na^+-K^+ exchange pump, ouabain, reduces adenosine release presumably by reducing ATP consumption. Reproduced from [118]. d) Conceptual summary of a proposed mechanism for activity dependent adenosine release based on findings from [118]. Excitatory activity for example via glutamatergic synaptic inputs and spiking results in an influx of Na^+. This is then pumped out by the Na^+-K^+ exchange pump (NKA) which consumes ATP. The resulting ADP is then

converted to AMP and adenosine by adenylate kinase (AK) and the 5’ nucleotidase (5’-NT) and the adenosine can cross the membrane via an equilibrative transporter (ENT).